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Isolation and identification of antioxidants components From Cumin seed (*Cuminum cyminum)*

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Abstract

Natural materials are complicated compositions; therefore, a rapid screening of the active antioxidants is being challenged in the literature. Different polarity solvents were applied to isolate four fractions (F1, F2, F3 and F4) of methanolic extract of *Cuminum cyminum*. Their anti-oxidative properties were tested using radical scavenging and FRAP assays. F3 (with IC_{50}=0.006mg/mL and FRAP= 521.95 mmolFe^{2+}/L) was significantly the most active fraction. Guided isolation through bio-autography on TLC using 1, 1- diphenyl- 2 – picryl-hydrazyl radical (DPPH) as a detection reagent led to the isolation of two antioxidant compounds from F3. F3 was injected to a preparative HPLC with the proper mobile phase (acetonitril: methanol/ water) and isolated two main compounds. These compounds were identified as Luteolin 7 glugoside and Apigenin 7 glucoside by means of ^{1}HNMR and ^{13}CNMR and compare them with references.

Key words: Antioxidant, Apigenin, *Cuminum cyminum,* Luteolin.

Introduction

A growing amount of evidence has shown that free radicals such as superoxide radical ($O\bullet^{-}_{2}$), hydroxyl radical (OH•), peroxyl radical (ROO•), and nitric oxide radical (NO•), attack biological molecules, such as lipids, proteins, enzymes, DNA and RNA. They play an important role in several pathological processes, such as cancer, atherosclerosis, and negative cellular changes associated with aging (Fisch *et al*., 2003; Nakman *et al*., 2003; shon, *et al*., 2003; Valentao, *et al*., 2002). Antioxidants are effective in protecting the body against these degenerative events. Synthetic antioxidants, such as butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) that are commonly used in lipid-containing food have safety and toxicity problems (Amarowicz *et al*., 2000; Howell, 1968). There is an increasing interest to seek for antioxidants naturally present in vegetables, fruits and functional herbs. Natural materials are complicated compositions therefore a rapidly screening of the active antioxidants is being challenged. A crude natural product extract is generally an extremely complicated mixture of several compounds possessing varying chemical and physical properties. The fundamental strategy for separating these compounds is based on their physical and chemical properties that can be effectively separated them into various chemical groups (Amarowicz *et al*., 2000).

Plant natural products are usually extracted with solvents of increasing polarity, for instance, at first extracted with petroleum ether, dichloromethane followed by more polar solvents, i.e., ethyl acetate. Methanolic extracts of plant materials contain a wide range of polar and moderately polar compounds (otsuka, 2006). By virtue of the co solubility, many compounds, which are insoluble individually in pure state in methanol can be extracted quiet easily with these solvents. Herein we use an activity guided

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purification of natural antioxidants from methanolic extract of cumin seed (*Cuminum cyminum*) by partitioning between insolvable solvents (Satti *et al*., 2009).

Cumin seed L. is widely used as a spice. Crushed cumin seeds are used as a condiment in a variety of dishes. The proximate composition of the seeds indicates that it contains fixed oil (10%), protein, cellulose, sugar and other mineral elements (Winton, 1939). Cumin seed contains essential oil (2%–5%) that imparts the characteristic aroma to the seeds and the physicochemical properties of it have been reported (Guenther, 1950).

Phenolic compounds present in spices with natural antioxidant properties have been studied for substitution of synthetic antioxidants. Compare to many traditional medicinal plants, little is known about the active antioxidant matter of Cumin seed. Active principles of cuminaldehyde (cumin) are reported to inhibit lipid peroxidation (Shobana and Akhilender, 2000) and it has been reported that the ether extract of cumin seed inhibits biosynthesis of eicosanoids (Satti *et al*., 2009) which are a pain mediators. Modulating effects of cumin seed was explored and its active compound, immuno-modulatory properties were evaluated in normal and immune- suppressed animals (Chauhan, et al., 2009). A preliminary study of cumin seed chemical composition was made on its essential oil had shown the presence of flavonoid compounds (Gachkar et al., 2007; Li *et al*., 2009; Behera, *et al*., 2004) which act as antioxidants in edible oils (Vekiari *et al*., 1993). Einafshar (2012) showed that Cuminum cyyminum has antioxidative effects in both bulk amd emulsion systems.

In the present study an activity guided purification was conducted to isolate the free radical scavenging compounds from Cumin seed, the bioactive constituents from the seeds of *Cuminum cyminum* was isolated and quantificated and their chemical compositions identified.

Materials and Methods

Materials

Cumin seeds were obtained from international company of Pegah Saffron and Zire Green Gold (Mashhad, Iran). Refined, bleached, and deodorized sunflower oil (SFO) with no added antioxidant was supplied by Shadgol (Nishbour, Iran). All chemicals and solvents used in this study were of analytical reagent grade and supplied by Merck and Sigma Chemical Companies.

Plant material

The sample seeds were dried in an cabin drier at 55°C for 2 days, ground into a fine powder in a mill (Mulinex Depose-Brevete S.G.C.G., France). The powders were extracted with methanol (1:20 w/v) by agitation in a dark place at ambient temperature for 48 h. The solvent was evaporated in vacuum at 40ºC. The desolventized extract (DEx) stored at -18ºC until use.

Extraction and isolation of antioxidant compounds

60 mg the methanolic extract of *Cumin seed* was dissolved in 100 ml methanol and extracted thrice using 100 ml petroleum ether under stir for 10 minutes. The upper phase of petroleum ether, contains nonpolar compounds such as lipids, chlorophyls and so on. This process is sometimes referred to as defatting. Although methanol and petroleum ether are not completely miscible, they are miscible to some extent. A small amount of water is added to methanol to obtain a 95% aqueous methanolic solution to get two distinct layers with similar volumes. Petroleum ether in the upper phase was removed by evaporation at 50°C under vacuum to give a residue named F1. The lower phase is partitioned in Dichloromethan and upper phase in ethylacetate successively and named F2 and F3 respectively. Less polar compounds are present in the dichloromethane soluble fraction and polar compounds, probably up to mono-glycosides, in the ethylacetate. The methanol fraction contains polar compounds mainly glycosides named F4.

The conventional spectrophotometric DPPH• scavenging capacity assay

The radical-scavenging activity was evaluated according to Chung *et al*. Two ml of the methanol solution of F1, F2, F3 and F4 were mixed with 1 ml of 0.5 mM DPPH• methanol solution and 2 ml of 0.1 M sodium acetate buffer (pH 5.5). After shaking, the mixture was incubated at room temperature in the dark for 30 min, and then the absorbance was measured at 517 nm using a Shimadzu UV-160A spectrometer, Kyoto (Japan). The IC_{50} value was determined as the concentration of each sample required to give 50% DPPH• radical scavenging activity.

FRAP test

Acetate buffer (0.3 M, pH 3.6) was prepared by dissolving 3.1 g $C_2H_3O_2Na.3H_2O$ and 16 ml of acetic acid in 1 l of distilled water. TPTZ (2, 4, 6 -tripyridyl-S-triazine) solution was prepared by dissolving 23.4 mg of TPTZ in 7.5 ml of 40 mM HCl solution. Ferric solution (20 mM) was prepared using $FeCl_3.6H_2O$. The final working FRAP reagent was prepared freshly by mixing acetate buffer, TPTZ and ferric solutions at a ratio of 10:1:1.

In brief, 900 µl FRAP (ferric reducing-antioxidant power) working reagent was mixed with 90 µl distilled water and was warmed to 37 ºC in a water bath. The reagent control reading was recorded at 595 nm, followed by adding 30 µl of sample solutions (100 mg in 10 ml of n-hexane). The absorbance was taken at 595 nm, against the control solution. A standard curve was prepared using different concentrations of $FeSO_4.7H_2O$ (200–2000 µmol/l). All solutions were freshly prepared. The results were expressed in mmol Fe^{2+}/l (Capannesi, et al., 2000).

HPLC preparative analysis

F3 methanol solution (1.0 mg/mL) was filtered through cellulose acetate membrane filter (0.45 mm, Anpu Co.,Shanghai, China) prior to HPLC analysis. 1 ml aliquot of the filtrate of concentration 3mg ml^{-1} in methanol was injected into a Wellchrom KNAUER HPLC, with reverse column (C18, 250 ×4.6 mm i.d., 5 mm), HPLC pump: KNAUER k-1001, UV detector: KNAUER k-2600, and eluted with a mobile phase containing solvent A (methanol) solvent B (acetonytril) and solvent C (water) (90:5:5) iso-cratically pumped at a flow rate of 1.0 mL/min. In the chromatogram the peaks of interest were observed at t_R 22.5 min and 27 min, detection was at 250nm. Compounds were collected in a clean, weighed flask from 21.5 to 25 min at room temperature under a room temperature. Multiple preparative HPLC separation was done and fractions for two peaks combined.

Processing of the collected fractions

The solvent was removed from the samples at 20°C with N_2 gas and the residual aqueous phases were shell-frozen on dry ice prior to freeze-drying. The freeze-dried samples were stored at -20°C until NMR analyses were performed. Complete removal of solvent was done by freeze drier and resulted yellow and yellow fade compounds powder.

NMR identification of compounds

NMR spectroscopy experiments on the compounds were performed on a Bruker Avance 500 at 500 MHz for ^1H NMR and ^{13}C NMR with CD_3OD and DMSO as solvent in 25 centigrade.

TLC bioautography analysis

An aliquot of AMS methanol solution (1 mg/mL, 3 mL) or individual pure isolate methanol solutions (1.0 mg/mL, 2 mL) was directly deposited (as spots or bands) onto the TLC plates. TLC plates were developed in a presaturated solvent chamber with ethyl acetate- acetonytril- formic acid- water- ethyl formiate (7:1:1:1.1:12) as developing reagents until the solvent front reached 1 cm from the top of plates. The developed TLC plates were then removed from Fig. 1. TLC plates stained with 2.54 mM DPPH_ solution in methanol, and visualized (A) under visible light, (B) under UV 254 nm, and (C) under UV 366 nm. Three microlitres Silica gel 60 F254 TLC plates (Merck, Germany) were used for TLC

bioautography analysis.

Result and discussion

DPPH$^{\bullet}$ scavenging activity and FRAP tests of isolates

The conventional spectrophotometric DPPH$^{\bullet}$ scavenging capacity assay was first used to screen the potential antioxidant of F1, F2, F3 and F4.The DPPH$^{\bullet}$ scavenging activities of all the fractions were estimated using the conventional spectrophotometric DPPH$^{\bullet}$ scavenging capacity assay. table.1 shows the quantities related to the DPPH radical-scavenging activities of the F1, F2, F3 and F4 compare to that of BHT (butylated hydroxy toluen) as an synthetic antioxidant. All fracions except F3 showed DPPH radical-scavenging activities extremely lower than that of BHT (F3=0.006, BHT=0.007, F1=1.47, F2=0.119 and F4=0.24 mg mL^{-1}). F3 showed significant DPPH$^{\bullet}$ scavenging activities which were similar to that of BHT.

Table 1. The DPPH radical-scavenging activity (IC_{50}) and ferric reducing-antioxidant power (FRAP) of the total extract and four fractions of *Cumin seed*. Diethylether fraction (F1), dichloromethane fraction (F2), Ethyl acetate fraction (F3) and methanolic fraction (F4).τ

Antioxidant matter (100 ppm)	IC_{50} (mg/mL)	FRAP (mmol Fe^{2+}/L)
Total extract	0.74 ± 0.10 b	459.46 ± 0.81e
F1	1.47 ± 0.03 a	229.63 ± 0.28 f
F2	0.119 ± 0.003 d	495.52 ± 0.37 d
F3	0.006 ± 0.001 f	521.95 ± 0.91 b
F4	0.24 ± 0.002 e	500.82 ± 0.69 c
BHT	0.007±0.002 f	1955.72±0.98 a

τ Means ± SD (standard deviation) within a column with the same lowercase letters are not significantly different at $P < 0.05$

The antioxidant activity decreased as follows: F3> F2> F4 > F1

Many similarities were found to exist between the results of the FRAP test and those of the DPPH radical-scavenging activity assay. Table 1 demonstrates that the reducing power of F3 (521.95 mmol/L) was significantly lower than that of BHT and higher than those of total extract (459.46 mmol/L) and other fractions. The chelating abilities of F3 on ferrous ion were good as shown by their low IC_{50} value (0.006 mg/mL). This indicates that the chelating activity of F3 in metal ion may play an important role in their antioxidant activity.

In the present study the F3 was selected for further purification, since less is known about the antioxidants components in the seeds of Cumin seed.

Isolation of antioxidant compounds

F3 fraction was then monitored by a TLC bio-autography method to guide the separation procedure because this method gives quick access for detection and localization of the active compounds in a complicated plant extract (Tasdemir, *et al.*, 2004). In this method, the DPPH$^{\bullet}$ scavenging activity was observed visually as white yellow spots on a purple background. Fig. 1-A shows the profile of the antioxidant components in the F3 fraction of the methanolic extract of Cumin seed under visible light. At least two spots (such as a and b) in the chromatograms were observed to have DPPH$^{\bullet}$ scavenging activities. In addition, the same stained TLC plate was also inspected under UV 254 and 366 nm.

Note that the antioxidant spots shown in Fig. 2A were also observed in those of Fig. 2B and C. However, it needs to be pointed out that the different detection sensitivities observed among Fig. 2 A–C were due to the diverse nature of the anti-oxidative compounds. For example, spot (a) was easily better visualized under 254 and 366 nm than that under visible light.

The F3 fraction was directly subjected to a preparative HPLC C18 column and eluted iso-cratically with methanol, acetonytril and water (90:5:5). Finally, two compounds were separated and identified, the structures of which are Apigenin-7-*O* glucoside and Luteolin- 7- *O* glucoside presented in Fig 2

and 3.

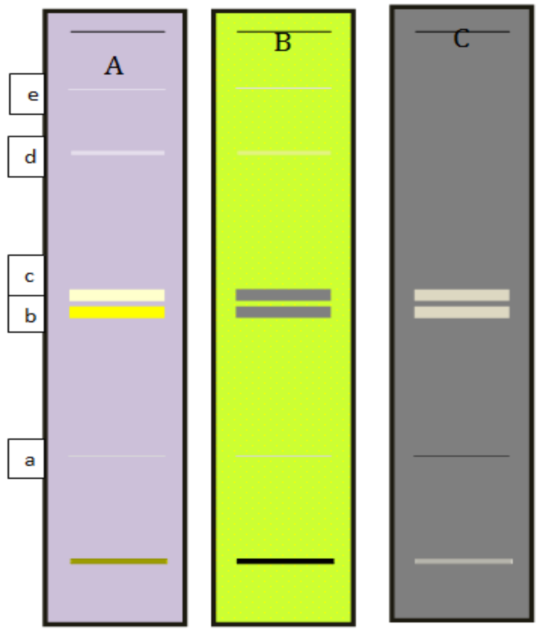


Fig. 1. TLC plates stained with 2.54 mM DPPH_ solution in methanol, and visualized (A) under visible light, (B) under UV 254 nm, and (C) under UV 366 nm. Three microlitres of the 80% methanol extract (AMS, 1 mg/mL) of *Cuminum cyminum* was applied as dots on TLC layer. The spots marked with b and c indicate compounds with DPPH_ scavenging activities.

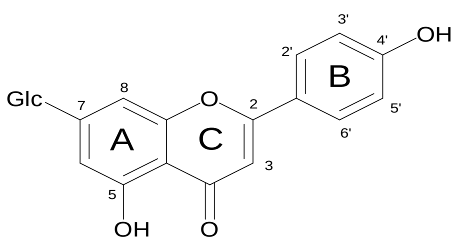


Fig 2. Apigenin-7-*O* glucoside

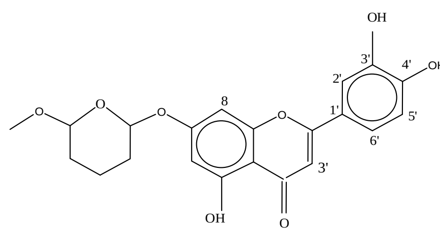


Fig 3. Luteolin- 7- *O* glucoside

Structure determinations of isolated compounds

The purity and identity of the compounds were established by use of spectroscopic techniques (^{1}H and ^{13}C NMR) and by comparison with the literature data. Compound 1 was also obtained as a yellow powder, and gave a positive reaction with natural products plus poly ethylen glycole reagent, probably indicating a flavonoid nature. Its UV spectrum was consistent with that of a flavonoid with maxima at 252, 265, and 347 nm (Gonza´ lez and Molina, 2006) A direct comparison of ^{1}H, ^{13}C NMR data (Table 2) with the reported data (Shen et al., 2004; Li *et al.*, 2007) led to identification of 2 as Luteolin-7-Glucosyde. This is the first report on the isolation of luteolin-7 –*O* glucoside from Cumin seed.

Compound 2 was obtained as a pale yellow powder, and showed characteristic flavonoid reaction with natural products plus poly ethylen glycole reagent. Its detailed UV (MeOH, λmax 265, 335 nm), 1H and 13C NMR data (Table 3), negative ESI MS data (m/z 269 [M_ H]_), agreed well with the previous reported data (Shen et al., 2004; Li *et al.*, 2007). Therefore, this compound was

identified as Apigenin-7-*O* Glucoside, and isolated from Cumin seed for the first time.

Table 2. NMR data of compound 1

Position	^{1}H-NMR data		^{13}C-NMR data	
	Compound 1	Ref [23,24]	Compound 1	Ref [23,24]
2	-	-	165.8	165.8
3	6.63,s	6.63,s	103.7	103.7
4	-	-	183.1	183.1
5	-	-	161.8	161.8
6	6.44, d,1.8H2	6.44, d,1.8H2	100.5	100.5
7	-	-	163.9	163.9
8	6.83,d,J=1.8H2	6.83,d,J=1.8H2	95.8	95.8
9	-	-	158	158
10	-	-	106.3	106.3
Glucose	-	-	122.6	122.6
1'	7.43,bs	7.43,bs	113.8	113.8
2'	-	-	146.3	146.3
3'	-	-	150.4	150.4
4'	6.95,d,J=8H_2	6.95,d,J=8H_2	116.5	116.5
5'	7.41,d,J=8H_2	7.41,d,J=8H_2	120.4	120.4
6'	-	-		
1"	5.1,d,J=7.8	5.1,d,J=7.8	100.7	100.7
2"	3.49,t,J=9H_2	3.49,t,J=9H_2	73.8	73.8
3"	3.56,t,J=9H_2	3.56,t,J=9H_2	76.8	76.8
4"	3.42,t,J=9H_2	3.42,t,J=9H_2	70.3	70.3
5"	360,m,	360,m,	77.4	77.4
6"	3.68,dd,J=12.2,5.6 H_a 3.85,dd,J=12.2, 1.8H_b	3.68,dd,J=12.2,5.6 H_a 3.85,dd,J=12.2, 1.8H_b	61.3	61.3

Table 3. NMR data of compound 2

Position	^{1}H-NMR data		^{13}C-NMR data	
	Compound 2	Ref[23,24]	Compound 2	Ref[23,24]
Aglycon	-			
2	-	-	166.77	164.7
3	6.8, S	6.8,S	104.14	103.4
4	-	-	184.08	182.4
5	-	-	162.93	161.9
6	6.5, S	6.5, d, J=2 Hz	96.07	95
7	-	-	164.81	163.1
8	6.6, S	6.6, d, J=2 Hz	101.19	104.7
9	-	-	158.96	157.3
10	-	-	107.1	105.7
1'	-	-	123.08	121.2
2'	7.9, d, J=8.0 Hz	7.9, d, J=8.8 Hz	129.64	128.9
3'	6.9, d, J=8.5 Hz	6.9, d, J=8.8 Hz	117.05	116.4
4'	-	-	162.93	161.4
5'	6.9, d, J=8.5 Hz	6.9, d, J=8.8 Hz	117.05	116.4
6'	7.9, d, J=8.0 Hz	7.9, d, J=8.8 Hz	129.64	128.9
Glucose	-			
1"	5.2, d, J=7H_2	5.2, d, J=7 Hz	101.64	99.9
2"	3.4 -4	3.4 -4	73.9	73.9
3"			75.2	75.2
4"			71	71
5"			76.1	76.1
6"			66.7	66.7

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جداسازی و شناسایی ترکیبات دارای خواص آنتی اکسیدانی زیره سبز (*Cuminum cyminum*)

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چکیده

مواد طبیعی ترکیبات پیچیده‌ای هستند که استخراج سریع مواد فعال آنتی‌اکسیدانی از اهمیت خاصی برخوردار است. برای جداسازی چهار جزء (F1، F2، F3 و F4) موجود در عصاره متانولی زیره سبز از حلال‌هایی با قطبیت متفاوت استفاده شد. خواص آنتی‌اکسیدانی اجزاء فوق، با استفاده از آزمـون تعیین قدرت‌گیرندگی رادیکال و آزمون FRAP تعیین شد. جزء F3 با (میلی-گرم بر میلی لیتر) IC500/006= و (میلی مول آهن 2+ در لیتر) 95 /521 FRAP= فعال‌ترین جزء بود. با استفاده ازکروماتوگرافی لایه نازک (TLC) و ارزیابی قدرت‌گیرندگی رادیکال آزاد (DPPH) دو ترکیب دارای خاصیت آنتی‌اکسیدانی از جزءF3 استخراج شد. به‌منظور تعیین ترکیب شیمیایی این دو جزء با استفاده از کرومـاتوگرافی تهیـه‌ای (HPLC preparative) بـه همراه فاز متحرک مناسب (استونیتریل: متانول/ آب) مقدار لازم از این دو ترکیب استخراج شد و با استفاده از HNMR و CNMR و مقایسه با منابع موجود، دو ترکیب لوتئولین 7 گلوکوزید و آپیژنین 7 گلوکوزید شناسایی گردیدند.

واژه‌های کلیدی: آنتی‌اکسیدان، آپیژنین، زیره سبز، لوتئولین.

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